

Application of AMP-Analogues
as General Ligands in
Affinity Chromatography
of Dehydrogenases

by
Peter Brodelius

Lund 1975



APPLICATION OF AMP-ANALOGUES AS GENERAL LIGANDS IN AFFINITY
CHROMATOGRAPHY OF DEHYDROGENASES

av

Peter Brodelius
Civ. ing., Vrm1

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To my parents
To Anna, Cecilia and Carl

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This thesis is based on the following publications, which are referred to by Roman numerals in the text.

- I. Affinity Chromatography of Enzymes on an AMP-Analogue: Specific Elution of Dehydrogenases from a General Ligand.
R. Ohlsson, P. Brodelius and K. Mosbach (1972) *FEBS Lett.* 25, 234-238
- II. The Synthesis of Three AMP-Analogues: N⁶-(6-Aminohexyl)-adenosine 5'-Monophosphate, N⁶-(6-Aminohexyl)-adenosine 2',5'-Bisphosphate, and N⁶-(6-Aminohexyl)-adenosine 3',5'-Bisphosphate and Their Application as General Ligands in Biospecific Affinity Chromatography.
P. Brodelius, P.-O. Larsson and K. Mosbach (1974) *Eur. J. Biochem.* 47, 81-89
- III. Separation of the Isoenzymes of Lactate Dehydrogenase by Affinity Chromatography Using an Immobilized AMP-Analogue.
P. Brodelius and K. Mosbach (1973) *FEBS Lett.* 35, 223-226
- IV. Determination of Dissociation Constants for Binary Dehydrogenase-Coenzyme Complexes by (Bio)Affinity Chromatography on an Immobilized AMP-Analogue.
P. Brodelius and K. Mosbach, submitted for publication.
- V. The Utilization of Immobilized Substrate/Product in Affinity Chromatography. A Model Study Using α -Chymotrypsin.
P. Brodelius and K. Mosbach (1972) *Acta Chem. Scand.* 27, 2634-2638

INTRODUCTION

Conventional methods for the purification of proteins depend on physico-chemical properties of the various proteins (charge, size, shape, hydrophobicity etc.) in a mixture. These differences are, however, often very small and complete separation of the proteins is difficult to achieve. Many chromatographic steps are required for a good purification. These methods are therefore laborious and the yield of purified protein is frequently low.

In affinity chromatography (or biospecific adsorption), on the other hand, often only one chromatographic step is needed to obtain a good purification of the desired protein. It is thus a relatively rapid method with often a very good yield. As the name biospecific adsorption implies, the method exploits the unique natural biological specificity between proteins and their ligands (substrate, inhibitor, cofactor or effector etc.). The ligand is immobilized on an insoluble support, this derivative is packed in a column and a mixture of proteins is passed through the column. Any protein(s) which exhibit affinity for the immobilized ligand will be retarded or adsorbed, while other proteins will pass unretarded through the column. The formation of the protein-ligand complex is reversible and the elution of the adsorbed protein(s) can be achieved by altering the composition of the buffer.

An early example of affinity chromatography was reported in 1953 by Lerman (1). Tyrosinase was purified with phenolic ligands which had been immobilized on p-diazobenzyl cellulose. However, the lack of a suitable insoluble support hindered the development of the method for several years. When a method for the activation of polysaccharides by cyanogen halides was presented (2, 3), beaded agarose was soon taken up as a suitable support for affinity chromatography.

Since then the technique has received increasing attention and it has become a widely used technique for the purification and separation of proteins (4-10). Some applications of affinity chromatography are summarized in *Table 1*.

Table 1. Some applications of affinity chromatography.

Purification of enzymes

antibodies

antigens

transport proteins

receptor proteins

Separation of isozymes

denaturated material from biologically active material.

Concentration of dilute protein solutions

Mechanistic and kinetic investigations on enzymes

GENERAL CONSIDERATIONS ON AFFINITY CHROMATOGRAPHY

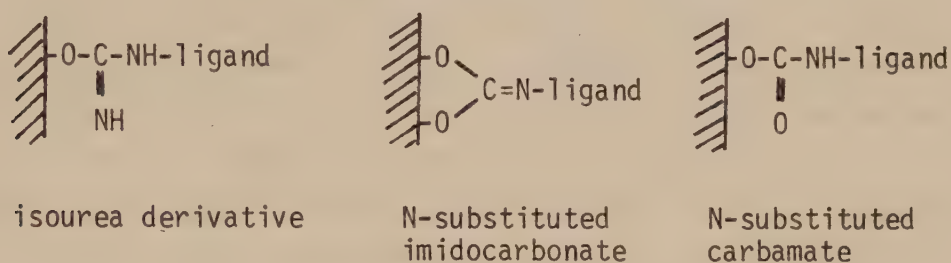
1. The solid support

As mentioned in the introduction the lack of a suitable insoluble support hindered the development of affinity chromatography for many years. There are several requirements for an ideal support. It should consist of uniform and spherical particles with good flow properties and which are mechanically and chemically stable to the conditions of both immobilization of ligand and of adsorption and elution of sample. For the immobilization step it must possess chemical groups that can be activated or functionalized and at the same time it must interact very weakly with proteins to minimize nonspecific binding. Finally the support should be hydrophilic and form a loose porous network, which permits the passage of macromolecules through the particles.

During the last few years efforts have been made to develop new supports which fulfill these requirements. So far no such support has been synthesized. A variety of supports, however, have been used in affinity chromatography. Cellulose was utilized in some early work (11, 12), but poor flow properties and non-uniform structure prevented wider use of this support. Beads of both cross-linked polyacrylamide (13, 14) and porous glass (15, 16), which possess several of the requirements of an ideal support, have been used with success in affinity chromatography.

The most extensively used support in affinity chromatography is beaded agarose (a polysaccharide consisting of D-galactose and 3,6-anhydro-L-galactose). It has a very loose structure and a molecular exclusion limit of several million daltons. The gel particles are uniform and spherical with relatively good flow properties. Furthermore it readily undergoes substitution reactions with primary amines when activated with cyanogen halides (2, 3). It can also be activated with bifunctional agents such as bisoxirane (17) and divinylsulfone (18). Ligands can be coupled through hydroxyl or amino groups to the bisoxirane or the divinylsulfone derivatives.

The activation of agarose with cyanogen bromide is rapid and completed within a few minutes at pH 11. The initial step of the activation is the formation of a labile cyanate which can react with adjacent hydroxyls to form an imidocarbonate. This imidocarbonate reacts with primary amines. In this reaction three derivatives are formed (19):



The isourea and imidocarbonate derivatives may be expected to carry a positively charged nitrogen at physiological pH. This can result in nonspecific binding of proteins to the solid support.

2. The ligand

To obtain an operative affinity chromatography system, there are a few considerations to be made concerning the selection and immobilization of the ligand.

The ligand must possess chemical groups that can be modified for linkage to the solid support without abolishing or impairing the protein-ligand interaction. Furthermore, this interaction must be sufficiently strong to permit binding of the protein to the immobilized ligand. On the other hand a very strong interaction can lead to problems during elution.

If the ligand is attached directly to the solid support a very poor binding of the complementary protein is observed (20, 21). This is due to steric hindrance which can be reduced or eliminated by introduction of a spacer arm (20, 21). Thus, by interposing a hydrocarbon chain between the ligand and the solid support the ligand will be more accessible to the protein and the binding is thus in-

creased. The optimum length of the spacer arm varies with the protein to be purified but a length of 5-8 Å (corresponding to a hydrocarbon chain of five or six atoms) has been shown to be satisfactory in many cases (20-22).

There are in principle two methods for the introduction of the spacer arm. The first involves immobilization of the spacer arm followed by coupling of the ligand to the terminal of this arm. However, this method can generate a matrix containing charged groups if all the spacer arms are not substituted with ligands. The second method avoids this drawback by coupling of the spacer arm to the ligand before the immobilization. A more defined affinity resin is obtained by this method. An additional advantage of the latter method is the possibility of determining, in a soluble system, if the ligand-protein interaction is affected to any great extent by the derivatisation of the ligand. Furthermore it is important that the ligand is specifically immobilized at one point to ensure a uniform resin.

Applications of affinity chromatography to systems of very high affinity have revealed a continuous leakage of the immobilized ligand (23). It has been suggested that the liberation of ligand is due to a nucleophilic attack of the solvent on the linkage between ligand and agarose (24). Furthermore an approach to overcome this difficulty has been presented, which involves the utilization of a polyvalent spacer arm (e.g. polylysine or polyvinylamine)(25). It is believed that higher stability could be expected of such derivatives due to multipoint attachment of the spacer. However, in most cases this problem might be negligible, but it can be serious in other cases where it is essential to have absolutely no free ligand.

3. The adsorption process

The interaction between a protein (P) and a ligand (L) can be described by the equilibrium



The dissociation constant (K_{diss}) of the protein-ligand complex (PL) is given by

$$K_{\text{diss}} = \frac{[P][L]}{[PL]} \quad (2)$$

When the ligand is immobilized this constant is affected by several factors. It is increased by steric hindrance and decreased by nonspecific binding to the solid support. The dissociation constant is related to the adsorption energy ΔG by the expression:

$$\Delta G = -RT \ln K_{\text{diss}} \quad (3)$$

where R is the gas constant and T the absolute temperature. The adsorption energy is composed of two terms, one representing the specific interaction (ΔG_{sp}) and a second representing the nonspecific adsorption (ΔG_{nonsp}), i.e.:

$$\Delta G = \Delta G_{\text{sp}} + \Delta G_{\text{nonsp}} \quad (4)$$

ΔG_{nonsp} , which often contributes to the adsorption, should be minimized (to avoid nonspecific binding of proteins to the column) and in the ideal case it is equal to zero.

If the concentration of immobilized ligand in the packed column is in the order of 10^{-3} M (this is the case for most agarose derivatives) a dissociation constant of 10^{-4} M or less results in effective binding of the protein, as has been established empirically (26) and theoretically (27). When a sample containing the desired protein is applied to an affinity column it will be adsorbed as a concentrated zone at the top of the column if the interaction between the protein and the immobilized ligand is sufficiently strong and if the capacity of the column is high compared to the amount of applied sample.

4. The elution process

When contaminating proteins have been washed out of the column, elu-

tion of the adsorbed protein can be achieved by altering the composition of the buffer in such a way that the dissociation constant for the complex between immobilized ligand and adsorbed protein is increased, i.e. the left hand side of the equilibrium is favoured (eq. 1). The elution conditions should, however, be as mild as possible to avoid damaging the purified protein.

Nonspecific methods of elution include changes in pH (28,29), ionic strength (30,31) or temperature (32). In most cases it is sufficient to change one of these physical variables to effect elution. In some cases a better elution pattern is obtained by simultaneously altering two of the variables (33). By a gradient technique it is possible to achieve an additional separation effect if more than one protein is adsorbed on the column (34).

If more than one protein is adsorbed onto the column a good purification can be obtained by a specific elution, which can be achieved by including a competing ligand in the buffer (35, 36). For elution this ligand should be present in higher concentration than the immobilized ligand or display a higher affinity for the protein.

Elution in a batchwise manner (i.e. by transferring the affinity gel-protein complex to another vessel and diluting) can be utilized when the protein-ligand interaction is very strong. The apparent concentration of immobilized ligand is drastically reduced by dilution and elution is readily obtained (37).

Elution by denaturing agent (guanidine-HCl or urea) may be required when the protein has a very high affinity for the immobilized ligand (38).

5. Restrictions and complications in affinity chromatography

Not until recently have serious attempts been made to develop the theory of affinity chromatography (27, 39). The problems are mostly concerned with the adsorption and desorption in a heterogeneous system. Still no perfect model has been established.

The development of a new affinity system is largely empirical. It involves extensive experimentation to achieve an applicable system. In most cases a special system is developed for each individual protein to be purified. This often requires elaborate chemistry. This inconvenience has been limited by the introduction of general ligands (40) which have affinity for a group of proteins. The specificity has been preserved by specific elution of one protein at a time (I). This will be discussed in more detail in the next chapter.

Futhermore, some knowledge of the protein to be purified is required in order to select a suitable ligand and to find appropriate conditions for the immobilization. When working with a new enzyme the lack of such knowledge can be disadvantageous. A more or less general approach to overcome those initial problems has been illustrated by a model study (V), which is treated in a later section.

Nonspecific adsorption of protein to the solid support has previously been mentioned as a complication which regularly occurs in affinity chromatography. These effects are generally relatively weak and hence they only are of significance in systems with low affinity. This kind of adsorption can either be due to ionic or hydrophobic bonding.

Untreated agarose exhibits no ionic exchange properties. Charged groups can, however, be generated or introduced during the activation of the gel and/or coupling of the ligand (19). By addition of salt in the buffer these ionic exchange properties can be reduced or abolished. The biospecific binding of the protein to the immobilized ligand is also influenced by this treatment. Generally, the affinity is reduced. New methods for the activation and the coupling have been developed to eliminate such generation of charged groups on the solid support (17, 18).

Hydrophobic bonding results from interactions between a hydrophobic spacer arm and hydrophobic groups or areas on the protein molecule. The most obvious way to reduce or eliminate this kind of nonspecific binding is the introduction of a hydrophilic spacer arm. Such

an arm can be produced by incorporation of polar groups at regular intervals along the spacer arm (41).

The observed interactions between enzymes and immobilized hydrophobic ligands led to the development of a new purification technique, which has been termed hydrophobic chromatography. A number of alkyl and aryl agarose derivatives (42, 43, 44) and ω -aminoalkyl agarose derivatives (43, 45, 46) have been utilized for purification of enzymes by hydrophobic interactions.

GENERAL LIGANDS IN AFFINITY CHROMATOGRAPHY FOR THE PURIFICATION AND SEPARATION OF ENZYMES

1. Choice of ligand

As mentioned previously general ligands or group-specific adsorbents were introduced to eliminate the drawback of synthesizing a specific ligand for each protein to be purified. An ideal general ligand should exhibit affinity for a large number of enzymes or family of enzymes. A variety of substances have been utilized as general ligands and the most extensively used are the coenzymes and analogues of them. Thus pyridine nucleotides have been utilized for the purification of dehydrogenases (*Table 2*) and adenine nucleotides for the purification of dehydrogenases and kinases (*Table 2*). Transferases (47) and synthases (47, 48) have been purified on uridine nucleotides, and transaminases on pyridoxal phosphate analogues (49, 50). Other examples of coenzymes which have been utilized as general ligands are flavin nucleotides (10, 11), folate (51) and lipoic acid derivatives (15, 52). The specificity involved in affinity chromatography purification has been maintained by development of specific elution methods, which have made it possible to selectively elute one enzyme at a time (I).

In the present work two general ligands have been utilized, i.e. N^6 -(6-aminohexyl)-adenosine 5'-monophosphate (I, III, IV) and N^6 -(6-aminohexyl)-adenosine 2',5'-bisphosphate (II). These analogues have structural resemblance to the coenzymes NAD^+ and $NADP^+$, respectively (*Fig. 1*). They can thus exhibit affinity for enzymes utilizing these coenzymes. Furthermore, they can serve as effectors for some allosteric enzymes and as inhibitors of other enzymes. Summarizing, these analogues exhibit affinity for several groups of enzymes and therefore they are excellent as general ligands.

A variety of adenine containing nucleotide analogues have been synthesized for utilization as general ligands in affinity chromatography (*Table 2*).

Table 2. Adenine nucleotide analogues which have been used as general ligands in affinity chromatography.

immobilized derivative	point of attachment	reference
$\equiv \text{NH}(\text{CH}_2)_6\text{NHCOCH}_2\text{-NAD}^+$	N ⁶	(53)
$\equiv \text{NH}(\text{CH}_2)_6\text{NHCO}-\text{C}_6\text{H}_4\text{-N=N-NAD}^+$	C ⁸	(54)
$\equiv \text{NH}(\text{CH}_2)_6\text{NH-NAD}^+$	C ⁸	(55)
$\equiv \text{NH}(\text{CH}_2)_6\text{NH-NAD}^+$	ribose hydroxyl	(56)
$\equiv \text{NHNHCO}(\text{CH}_2)_4\text{CONHN=NAD}^+$	ribose	(57)
$\equiv \text{NH}(\text{CH}_2)_6\text{NHCOCH}_2\text{-NADP}^+$	N ⁶	(58)
$\equiv \text{NH}(\text{CH}_2)_6\text{NHCO}-\text{C}_6\text{H}_4\text{-N=N-NADP}^+$	C ⁸	(54)
$\equiv \text{NH}(\text{CH}_2)_6\text{NH-NADP}^+$	C ⁸	(59)
$\equiv \text{NHNHCO}(\text{CH}_2)_4\text{CONHN=NADP}^+$	ribose	(57)
$\equiv \text{NH}(\text{CH}_2)_6\text{NHCOCH}_2\text{-ATP}$	N ⁶	(60)
$\equiv \text{NH}(\text{CH}_2)_6\text{NHCO}-\text{C}_6\text{H}_4\text{-N=N-ATP}$	C ⁸	(59)
$\equiv \text{NHNHCO}(\text{CH}_2)_4\text{CONHN=ATP}$	ribose	(57)
$\equiv \text{NH}(\text{CH}_2)_6\text{-ADP}$	N ⁶	(61)
$\equiv \text{NH}(\text{CH}_2)_6\text{NHCOCH}_2\text{-ADP}$	N ⁶	(60)
$\equiv \text{NH}(\text{CH}_2)_6\text{NHCO}-\text{C}_6\text{H}_4\text{-N=N-ADP}$	C ⁸	(59)
$\equiv \text{NH}(\text{CH}_2)_6\text{NH-ADP}$	C ⁸	(61)
$\equiv \text{NHNHCO}(\text{CH}_2)_4\text{CONHN=ADP}$	ribose	(57)
$\equiv \text{NH}(\text{CH}_2)_6\text{-ADP}$	terminal phosphate	(61)
$\equiv \text{NH}(\text{CH}_2)_6\text{-2'5'5'-ADP}$	N ⁶	(II)
$\equiv \text{NH}(\text{CH}_2)_6\text{NH-2'5'5'-ADP}$	C ⁸	(59)
$\equiv \text{NH}(\text{CH}_2)_6\text{-5'-AMP}$	N ⁶	(62)
$\equiv \text{NH}(\text{CH}_2)_6\text{NHCOCH}_2\text{-5'-AMP}$	N ⁶	(60)
$\equiv \text{NH}(\text{CH}_2)_6\text{NH-5'-AMP}$	C ⁸	(55)
$\equiv \text{NHNHCO}(\text{CH}_2)_4\text{CONHN=5'-AMP}$	ribose	(57)
$\equiv \text{NH}(\text{CH}_2)_6\text{NH-2'-AMP}$	C ⁸	(55)
$\equiv \text{NH}(\text{CH}_2)_6\text{-cAMP}$	N ⁶	(62)
$\equiv \text{NH}(\text{CH}_2)_6\text{NH-cAMP}$	C ⁸	(62)

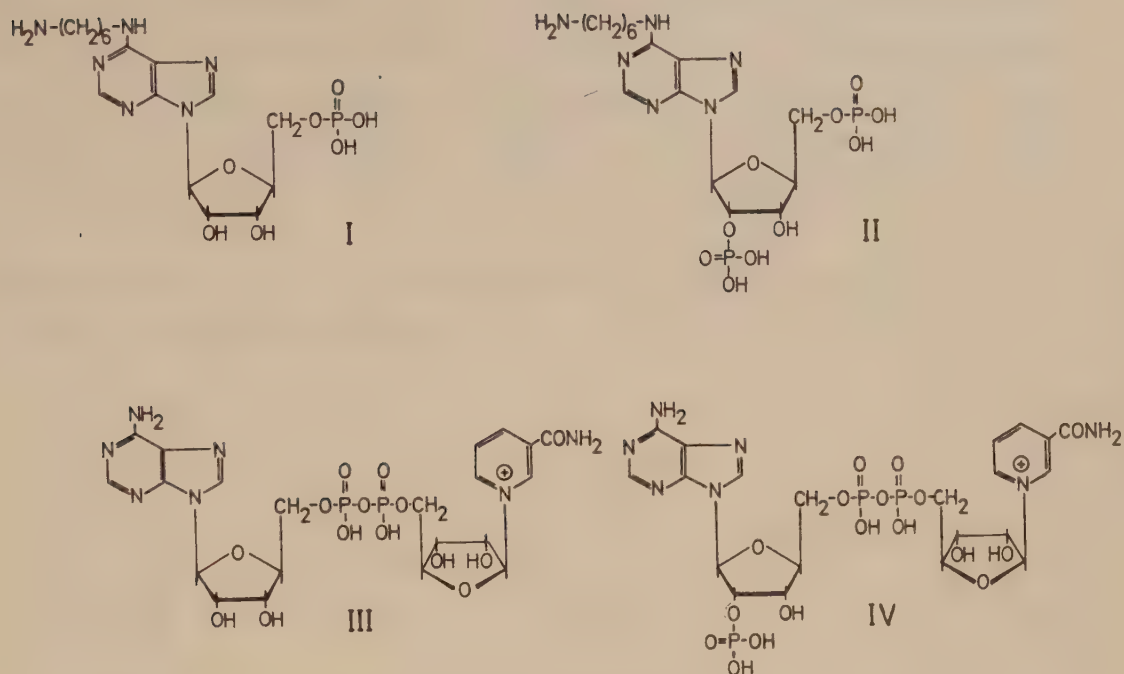
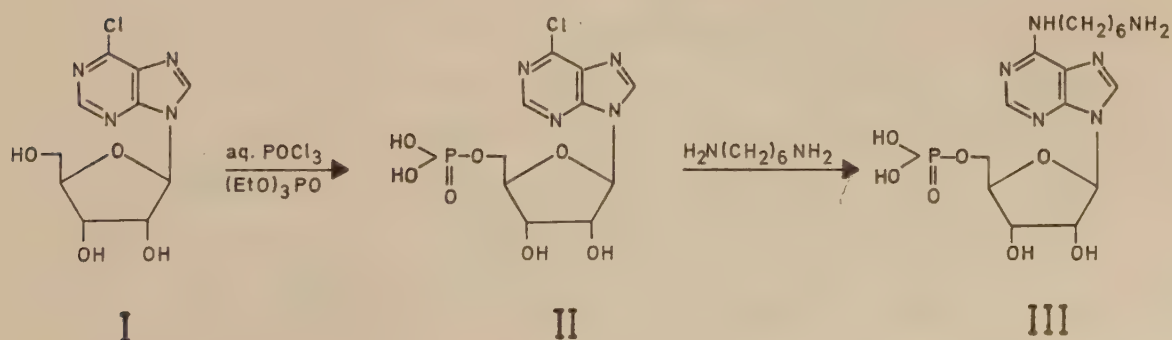


Fig. 1. Structure of N⁶-(6-aminohexyl)-adenosine 5'-monophosphate (I), N⁶-(6-aminohexyl)-adenosine 2',5'-bisphosphate (II), NAD⁺ (III) and NADP⁺ (IV).

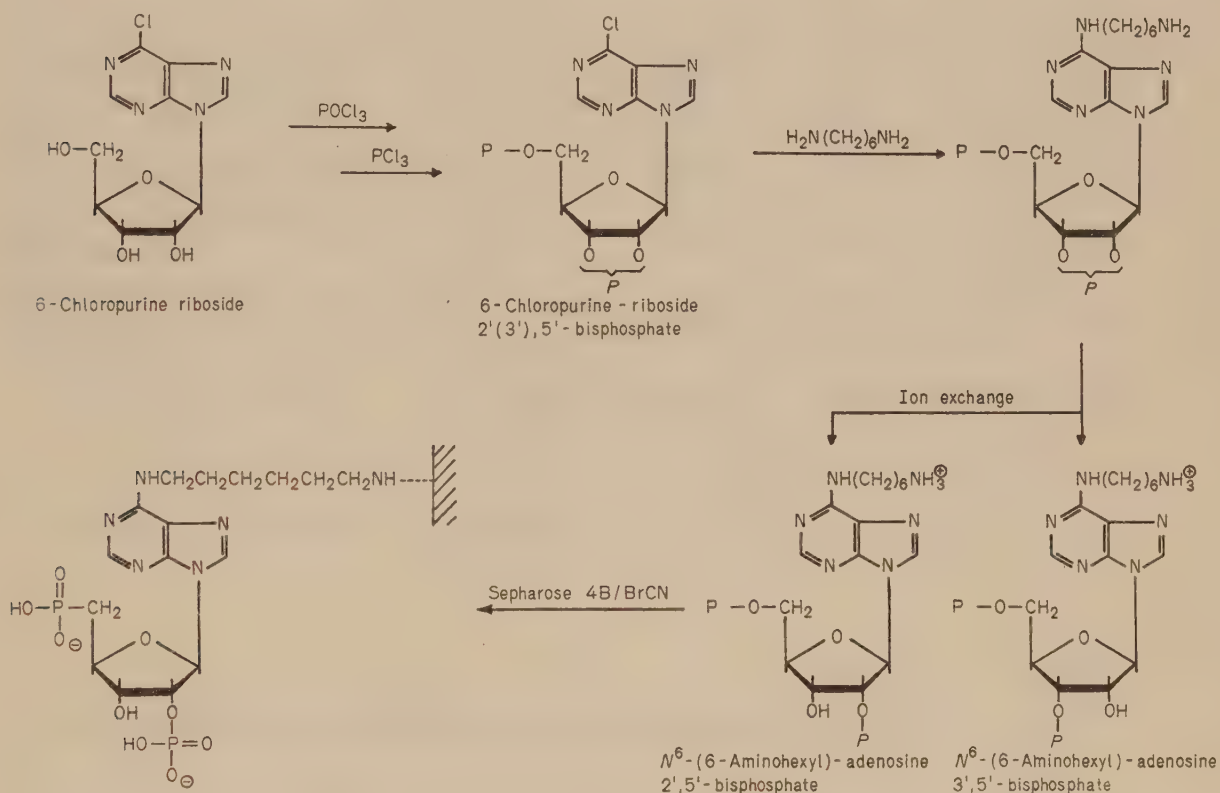
2. The synthesis of N⁶-(6-aminohexyl)-adenosine 5'-monophosphate and N⁶-(6-aminohexyl)-adenosine 2',5'-bisphosphate

The preparation of N⁶-(6-aminohexyl)-5'-AMP was performed as described by Guilford *et al.* (62):



6-Chloropurine riboside (I) is phosphorylated by aqueous phosphoryl chloride in triethylphosphate. The product (II) is treated with hexamethylene diamine to yield the N^6 -alkylated nucleotide (III). An alternative procedure for the synthesis of this analogue from 6-mercaptopurine riboside 5'-monophosphate has been reported (63).

The preparation of N^6 -(6-aminohexyl)-2',5'-ADP is analogous to the synthesis of the AMP-analogue (II).



The phosphorylation is carried out in two consecutive steps. First the primary hydroxyl (5'-position) is phosphorylated by phosphoryl chloride and in the second step the secondary hydroxyls (2'- and 3'-position) are phosphorylated by phosphorus trichloride. The resulting 2'(3')-phosphite moiety is oxidized to phosphate with gaseous chlorine. Treatment with hexamethylene diamine yields a mixture of N^6 -(6-aminoethyl)-2',5'-ADP and N^6 -(6-aminoethyl)-3',5'-ADP. These isomers are separated by chromatography on a column of Dowex 1-X2. The 3',5'-ADP-analogue is a part of coenzyme A and therefore is a potential general ligand for coenzyme A-dependent enzymes.

A similar procedure for the preparation of these two adenosine analogues has been reported (64). A disadvantage of this latter method, however, is the isolation of the intermediate, 6-chloropurine ribo-

Table 3. Inhibition of enzyme activities by AMP-analogues. The effect of N^6 -(6-aminohexyl)-5'-AMP (I), N^6 -(6-aminohexyl)-2',5'-ADP (II) and N^6 -(6-aminohexyl)-3',5'-ADP (III) on the activities of several enzymes was determined. The nucleotides were included in the standard assays. Rates are relative to AMP-analogue-free system (no inhibition = 1.00).

Enzyme	Source	Coenzyme used	Relative rate with added AMP-analogue		
			I ^a	II ^b	III ^c
Alcohol dehydrogenase	horse liver	NAD ⁺	0.53	1.00	1.03
Lactate dehydrogenase	beef heart	NADH	0.52	0.95	0.97
Isocitrate dehydrogenase	pig heart	NADP ⁺	1.06	1.00	0.97
Glucose-6-phosphate dehydrogenase	<i>Candida utilis</i>	NADP ⁺	0.89	0.60	0.88
6-Phosphogluconate dehydrogenase	<i>Saccharomyces cerevisiae</i>	NADP ⁺	1.07	0.61	1.00
Glutathione reductase	<i>Saccharomyces cerevisiae</i>	NADPH	0.95	0.13	0.77

^a 0.82 mM ^b 0.93 mM ^c 0.92 mM

3. Inhibition of some enzymes by the adenosine analogues

The inhibition properties of the synthesized analogues were investigated by including them in the standard assays for various enzymes. The results are summarized in *Table 3*. The NAD⁺-dependent enzymes were only inhibited by the AMP-analogue, while the NADP⁺-dependent were only inhibited by the ADP-analogue to any extent. It is possible by using such inhibition studies to predict the usefulness of the analogues as affinity adsorbents, since it has been shown that the inhibition and affinity properties are well correlated (II).

4. Coupling of the adenosine analogues to agarose

The adenosine analogues were immobilized on Sepharose 4B, which had been activated with cyanogen bromide (2, 3). The amount of immobili-

zed ligand was determined by total phosphate analysis on freeze dried samples (65). The ligand content varied between 45 and 200 μmol per g of dry polymer depending on the quantity of cyanogen bromide used in the activation of the gel.

5. Affinity chromatographic model studies on general ligands.

The effectiveness of immobilized N^6 -(6-aminohexyl)-5'-AMP as a general ligand is, as previously mentioned, a reflection of the fact that AMP is an inhibitor of a great number of both NAD^+ -dependent and other enzymes. When a number of enzymes are adsorbed on such a ligand, a successful separation depends on the degree of specificity that can be achieved during subsequent elution.

The technique of pulse elution has been applied to the separation of glyceraldehyde 3-phosphate dehydrogenase and lactate dehydrogenase using respectively NAD^+ and NADH as eluants (39). This method of elution can, however, involve considerable efforts before ideal elution conditions are evolved. Other highly selective methods of elution, some of which are more predictable in specificity, have been developed (1).

A linear gradient of NADH applied to a column containing adsorbed lactate dehydrogenase and glyceraldehyde 3-phosphate dehydrogenase separated them into two distinct peaks (1). The separation obtained by gradient elution is the result of two kinetic parameters, i.e. the enzyme-immobilized ligand interaction and the enzyme-eluant interaction. The dual operation of these affinity properties should ensure a good separation effect in gradient elution.

A gradient of NAD^+ separated glyceraldehyde 3-phosphate dehydrogenase, malate dehydrogenase and lactate dehydrogenase on the same immobilized AMP-analogue (66).

The formation of "dead-end" ternary complexes between enzyme, co-enzyme and substrate has been demonstrated for several NAD^+ -dependent dehydrogenases (67). This ability to form ternary complexes has been used for specific elution of dehydrogenases from the AMP-analogue column. By including NAD^+ and a substrate, substrate ana-

logue or inhibitor in the buffer at an appropriate concentration a ternary complex is formed and the enzyme is eluted. Using this method, a mixture of yeast alcohol dehydrogenase and beef heart lactate dehydrogenase were separated by elution with NAD^+ plus hydroxylamine (an inhibitor to alcohol dehydrogenase) and NAD^+ plus pyruvate respectively (1).

Another excellent example of the applicability of this method is the separation of lactate dehydrogenase and malate dehydrogenase on a column of immobilized ADP-analogue (66). Elution with a gradient of NAD^+ resulted in a very poor separation. If, however, either pyruvate or malate was added to the NAD^+ -gradient the two enzymes were completely resolved. When pyruvate was used lactate dehydrogenase was eluted first and when malate was used malate dehydrogenase was eluted first.

Other enzymes that have been specifically eluted by ternary complex formation are L-threonine dehydrogenase (68) and liver alcohol dehydrogenase (69).

Perhaps the most sophisticated method of specific elution is the utilization of a preformed adduct between NAD^+ and a substrate. The adduct is synthesized by base-catalyzed condensation of NAD^+ and the substrate (70). These adducts bind very specifically and strongly to the complementary dehydrogenase and are therefore excellent as eluants.

The oxidized NAD^+ -pyruvate adduct has been shown to be effective for the elution of lactate dehydrogenase from the AMP-column (1), while the reduced adduct has been shown to be even more effective (unpublished observation). A mixture of malate dehydrogenase, alcohol dehydrogenase and lactate dehydrogenase has been completely resolved by elution with reduced adducts (69).

The efficiency of the different eluant systems described has been demonstrated for the elution of ox heart lactate dehydrogenase (1), and they can be ordered in the following way:

$\text{NADH} > \text{reduced NAD-pyruvate adduct} > \text{oxidized NAD}^+\text{-pyruvate adduct} > \text{NAD}^+ \text{ plus pyruvate} > \text{NAD}^+ \text{ plus lactate} > \text{NAD}^+$

This order is easily correlated to the strength of the corresponding complexes. Thus the K_{diss} for the lactate dehydrogenase-NADH complex is the lowest ($0.39 \mu\text{M}$ (71)) and for the lactate dehydrogenase-NAD⁺ complex the highest (0.35 mM (72)).

N⁶-(6-Aminohexyl)-2',5'-ADP is a general ligand of similar broad applicability but specific for NADP⁺-dependent dehydrogenases. It has been shown to be useful in the purification of NADP⁺-dependent enzymes (II).

Mixtures of NAD⁺- and NADP⁺-dependent enzymes were applied to columns of immobilized 2',5'-ADP-analogue. The NAD⁺-dependent enzymes (alcohol dehydrogenase and lactate dehydrogenase) appeared in the void volume, while the NADP⁺-dependent (glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and glutathione reductase) were adsorbed to the immobilized ligand. These were subsequently eluted with pulses of NADP⁺ or NADPH.

The same mixtures were also applied to AMP-columns. In this case the NADP⁺-dependent enzymes appeared in the void volume, while the other were adsorbed. An exception was glucose 6-phosphate dehydrogenase, which was weakly bound to the AMP-gel. This is in line with the inhibitory action of the AMP-analogue (see *Table 3*).

These model studies clearly demonstrate a well expressed specificity of the respective enzyme for both ligand and eluant. Furthermore, they indicate that no significant nonspecific binding occurs. Isocitrate dehydrogenase (NADP⁺-dependent) is neither inhibited by the 2',5'-ADP-analogue (see *Table 3*) nor does it bind to the immobilized ligand (II). The enzyme, however, is adsorbed to 2',5'-ADP immobilized via the C⁸-position (59). This indicates that isocitrate dehydrogenase is more sensitive to derivation of the N⁶-amino group of the adenine moiety than the other dehydrogenases studied, and accordingly the enzyme cannot utilize deamino-NADP⁺ (73) or N⁶-substituted NADP⁺ (58) as coenzyme.

This difference in affinity for differently substituted analogues may reflect differences in the structure of the coenzyme binding sites of the various enzymes, and by comparative studies using dif-

ferently substituted analogues it should thus be possible to obtain information on the coenzyme binding site of an enzyme.

6. Purification of enzymes from crude extracts.

The capacity of an AMP-column has been tested for crude chicken heart lactate dehydrogenase (unpublished). A sample was continuously applied to the column until the lactate dehydrogenase activity of the eluate was the same as for the applied solution. A continuous leakage of enzyme was observed upon washing of the column with buffer. This indicated weakly adsorbed protein and therefore the column was washed with buffer containing 1 M NaCl. A large amount of lactate dehydrogenase was eluted and after further washing with buffer containing 1 M NaCl the activity of the eluate was zero. Application of a pulse of 10 mM NADH eluted more lactate dehydrogenase, which thus had been adsorbed strongly to the affinity resin. This elution profile indicates that the enzyme is adsorbed to the column by more than one mechanism. It is likely that the strongly adsorbed portion of the enzyme (approximately 30 % of the total amount of retained enzyme) represents the specific interaction between enzyme and immobilized ligand. A fraction of the immobilized ligand is, however, not completely sterically accessible to the enzyme and the weaker adsorption could thus be due to interactions between the enzyme and only a part of the ligand molecule. This weaker adsorption of lactate dehydrogenase could also be a result of protein-protein interactions. The initially specific adsorption of enzyme leads to a layer of protein which can serve as a platform for such interactions. When crude preparations are applied to affinity columns, nonspecific effects such as protein-protein interactions may be expected. In the experiment described approximately 0.5 mg of lactate dehydrogenase was adsorbed strongly (specifically) per ml of gel. When a pure enzyme is applied at least five times as much enzyme can be adsorbed specifically.

At saturation of the affinity column with lactate dehydrogenase only a few percent of the immobilized ligand is occupied. A reasonable conclusion from this observation is that when an enzyme molecule is bound to a ligand molecule it hinders several other ligand mole-

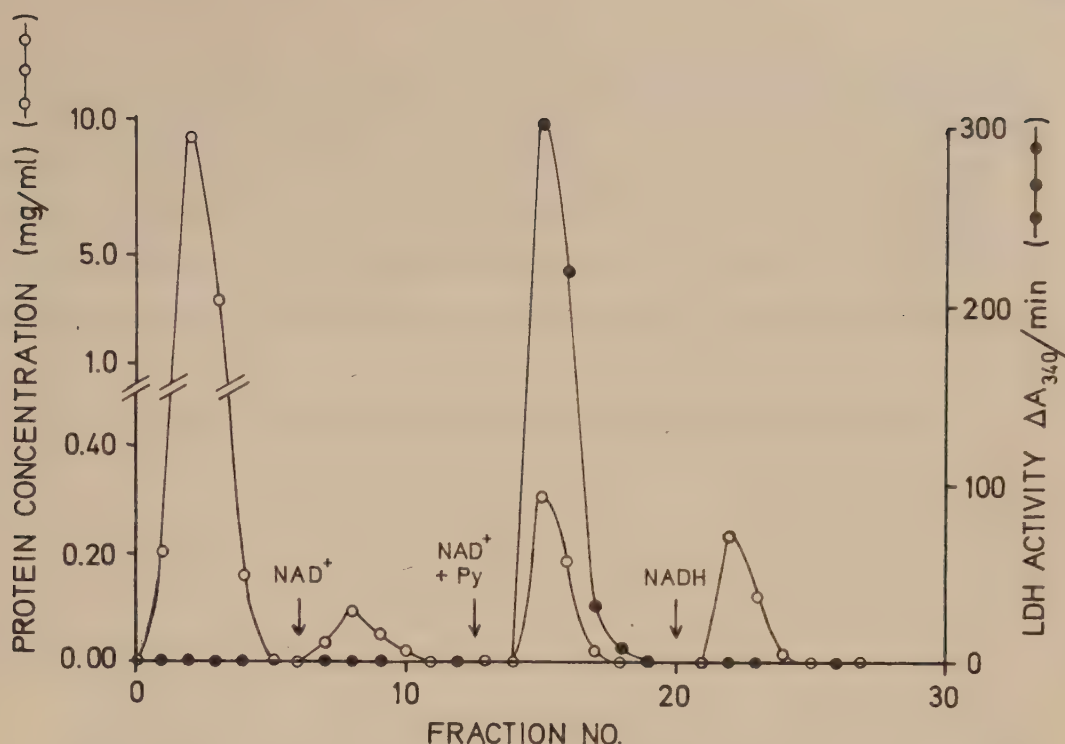


Fig. 2. Purification of lactate dehydrogenase from a crude extract of ox heart.

cules from interacting with further enzyme. In addition to this, as mentioned above, not all of the immobilized ligand is sterically accessible to the bulky protein molecule. Optimization of the ligand concentration might in many cases be valuable since affinity adsorbants containing a high ligand concentration are often used, whereas such high concentrations may not be necessary. Similar results have been reported for other calculations on the fraction of ligand which is operative during an affinity chromatography purification (74, 75).

To avoid overloading the column, care must be taken with the amount of protein applied. When very crude preparations are used a fractionation of the proteins by a conventional method before the affinity chromatography purification can increase the capacity of the columns considerably (76).

a. Purification of ox heart lactate dehydrogenase (I)

A crude extract of ox heart was applied to an AMP-column. The lac-

tate dehydrogenase was specifically eluted with NAD^+ and pyruvate as described above (*Fig. 2*). Before the elution an extra wash with NAD^+ was performed to elute other proteins which otherwise could interfere in the elution of the desired enzyme. The enzyme was purified 35 times with an almost quantitative recovery of activity. Polyacrylamide gel electrophoresis indicated a homogeneous protein.

b. Purification of human erythrocyte lactate dehydrogenase.

The purification was in principle performed as described for the ox heart enzyme, except that oxamate (an inhibitor of lactate dehydrogenase) was utilized instead of pyruvate. The purification was performed as follows (not published elsewhere):

Haemolysate (110 ml) was dialyzed against 0.1 M sodium phosphate buffer, pH 7.5 for 4 h, whereupon it was applied to an AMP-column (1x10 cm, containing 5 ml of gel). After application the column was washed with buffer (55 ml), buffer containing 0.4 M NaCl (130 ml) and buffer containing 0.5 mM NAD^+ (130 ml). Elution was achieved with buffer containing 0.5 mM NAD^+ and 10 mM oxamate. The fractions containing lactate dehydrogenase activity were pooled and the protein was precipitated by addition of ammonium sulphate to 70 % saturation. After centrifugation a suspension of human erythrocyte lactate dehydrogenase (0.7 ml) was obtained.

The results are summarized in *Table 4*. As can be seen a purification of approximately 15,500 times was obtained.

Table 4. Purification of lactate dehydrogenase from human erythrocytes.

step	total volume (ml)	total units	U/ml	protein (mg/ml)	specific activity (U/mg)
haemolysate	110	466	4.24	135	0.031
affinity chromatography	23	408	17.8	0.04	435
$(\text{NH}_4)_2\text{SO}_4$ -precipitation	0.72	420	583	1.2	486

This purification procedure clearly illustrates the applicability of affinity chromatography for the purification of enzymes present in very low concentrations.

AMP-Sepharose has also been used for the purification of lactate dehydrogenase from dog fish muscle on a preparative scale, and from human serum (69). Specific elution was in these cases obtained by including reduced NAD-pyruvate adduct in the buffer.

c. Purification of NADP^+ -dependent enzymes from a yeast extract (II).

A yeast extract (*Candida utilis*) was applied to a column of immobilized 2',5'-ADP-analogue. A number of NADP^+ -dependent enzymes were eluted by a linear gradient of NADP^+ . Cross-contamination of the enzymes occurred but it should be possible to improve the purification by rechromatography of the appropriate fractions on the affinity column.

In Table 5 the purification obtained of some NADP^+ -dependent enzymes are given. It is worth mentioning that the NAD^+ -dependent glutamate dehydrogenase was eluted in the void volume. This illustrates the possibility of determining coenzyme specificity by utilizing an appropriate analogue as adsorbent.

Table 5. Purification of some NADP^+ -dependent enzymes by affinity chromatography on immobilized N^6 -(6-aminohexyl)-2',5'-ADP.

enzyme	specific activity (U/mg)		purification (fold)
	crude extract	peak fraction	
glucose 6-phosphate dehydrogenase	1.11	208	187
glutamate dehydrogenase	0.57	20	35
glutathione reductase	1.12	63	56
6-phosphogluconate dehydrogenase	1.17	38	33

When a yeast extract (Baker's yeast) was applied to a column containing 8-substituted NADP^+ and subsequently eluted with a linear gradient of NADP^+ a slightly different elution profile was obtained (59), and the elution order of some enzymes was changed. This comparison shows that the relative affinity of the enzymes for a given ligand changes. The most striking difference was, however, isocitrate dehydrogenase, which was adsorbed to the C^8 -substituted NADP^+ -derivative, whereas it was not adsorbed to the N^6 -substituted ADP-analogue. This is in accordance with the previously discussed affinity properties of isocitrate dehydrogenase from pig heart. The yeast and pig heart enzymes are thus apparently similar in co-enzyme binding.

d. Purification of transhydrogenase from *Pseudomonas aeruginosa*.

The enzyme nicotinamide nucleotide transhydrogenase catalyzes the reaction:



The enzyme from *Pseudomonas aeruginosa* is allosterically regulated by 2'-AMP and NADPH as well as by other 2'-nucleotides (77). The reduction of NADP^+ by NADH is stimulated by these effectors and the stimulation is strongly influenced by Ca^{2+} (78). The affinity for the 2'-nucleotide is enhanced by Ca^{2+} . This phenomenon has been utilized for the purification of the enzyme from a crude extract of the bacteria (79). Application of the sample is performed in a buffer containing Ca^{2+} and elution is obtained by omitting the Ca^{2+} and by simultaneously increasing the pH. The purification was carried out as follows:

The bacteria cells were sonificated for 4x1 min in 0.1 M Tris-HCl buffer, pH 7.0. After centrifugation for 20 min at 20000xg the supernatant was made 0.1 M in mercaptoethanol and 5 mM in Ca^{2+} and applied to a column of immobilized 2',5'-ADP-analogue (0.8x10 cm, containing 3.5 ml gel), which previously had been equilibrated with the same buffer. Elution was achieved with 0.1 M Tris-HCl buffer pH 8.9, containing 0.1 M mercaptoethanol and 1 mM EDTA. The fractions containing transhydrogenase activity were pooled and concentrated by ultrafiltration. The concentrated solution was applied to a column of Sephadex G-200 (2x80 cm, containing 200 ml gel), equilibrated with 0.1 M Tris-HCl buffer pH 7.0 and 0.1 M mercaptoethanol. The transhydrogenase was eluted in the void volume.

Table 6. Purification of transhydrogenase from *Pseudomonas aeruginosa*.

step	total volume (ml)	total units	U/ml	protein (mg/ml)	specific activity (U/mg)
sonificate	286	1287	4.5	24.7	0.18
supernatant	242	1385	5.7	17.0	0.34
affinity chromatography concentrate	7	1170	167	0.81	206
Sephadex G-200	16	885	55.3	0.12	469

The results are summarized in Table 6. As can be seen a total purification of approximately 2,600 times was obtained with a yield of 70 %. Gelelectrophoresis indicated a pure and homogeneous protein.

The conventional purification procedure for this enzyme involves eight steps and a recovery of only 15 % is obtained (80). A comparison of these methods emphasize the advantages of affinity chromatography. The enzyme has also been purified on AMP-Sepharose (69) as well as on 8-substituted 2'-AMP (55).

Preliminary experiments have shown that the enzyme is allosterically activated when adsorbed to the column. The utilization of immobilized AMP as an activating agent has also been reported (81), when glycogen phosphorylase b, which has an absolute requirement for AMP, was adsorbed to a column of immobilized N⁶-(6-aminohexyl)-5'-AMP and a "permanently" activated enzyme was obtained.

The inhibition of transhydrogenase in submitochondrial particles from beef heart mitochondria by some adenosine analogues has been investigated. The results are summarized in Table 7. It should be pointed out that the mitochondrial enzyme differs in many aspects from the bacterial enzyme (82). It is for instance not allosterically regulated by 2'-nucleotides.

Table 7. Inhibition of transhydrogenase from beef heart mitochondria by adenosine analogues.

inhibitor	% inhibition
2'-AMP	46
N ⁶ -(6-aminohexyl)-5'-AMP	10
N ⁶ -(6-aminohexyl)-2',5'-ADP	72
N ⁶ -(6-aminohexyl)-3',5'-ADP	22

A K_i of approximately 0.3 mM for the 2',5'-ADP-analogue was estimated from these experiments. A preparation of mitochondrial transhydrogenase, solubilized by Triton X-100, was neither adsorbed nor retarded on a column of the immobilized 2',5'-ADP-analogue. A possible explanation of this is a layer of large detergent molecules on the protein surface, which makes it impossible for the immobilized ligand to "reach" the active site of the enzyme. However, affinity chromatography purifications of proteins solubilized by detergents have been successfully carried out using resins with comparatively long spacer arms (approximately 30 Å long) (83, 84).

7. Separation of lactate dehydrogenase isozymes (III).

In vertebrates, the NAD^+ -dependent enzyme lactate dehydrogenase is a tetramer which *in vivo* catalyzes the reduction of pyruvate to lactate. Two genetically determined subunits (H and M) exist which can combine to form five different tetramers, H_4 , H_3M , H_2M_2 , HM_3 and M_4 . Isozymes of mainly H-subunits predominate in aerobic tissues, such as heart and brain, while isozymes of mainly M-subunits are found in anaerobic tissues such as skeletal muscles and liver. Their kinetic properties are different and it has been suggested that the distribution of the isozymes is related to the metabolism of the various tissues. Furthermore, it has been established that the isozyme pattern can be altered by several diseases. It is therefore of importance for diagnosis to be able to confirm the isozyme pattern. Furthermore, pure isozymes are desired for structural and kinetic in-

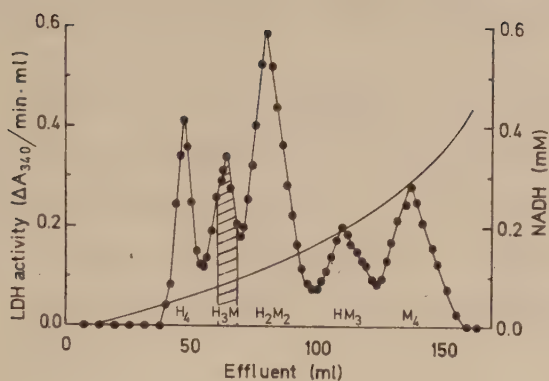


Fig. 3a. Elution of lactate dehydrogenase isozymes by a concave gradient of NADH from a column of immobilized AMP-analogue.

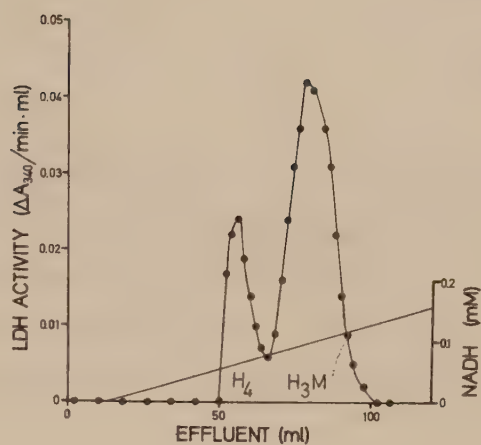


Fig. 3b. Elution of rechromatographed H_3M isozyme by a linear gradient of NADH from a column of immobilized AMP-analogue.

vestigations. To this end, ion exchange matrices and electrophoresis have been mainly used (85). The potential of the technique of affinity chromatography for such a purpose has been studied because of the general inherent advantage of this technique.

Thus, a mixture of the five bovine isozymes was applied to an AMP-column. As seen from *Fig. 3a*, on subsequent application of a gradient of NADH, five distinct peaks were obtained representing the five different isozymes as shown by gelelectrophoresis. A complete separation was not obtained, but to prove that it is possible, the fractions containing H_3M (corresponding to the hatched area in *Fig. 3a*) were pooled and rechromatographed on the same column after removal of NADH by dialysis. *Fig. 3b* indicates that pure H_3M could be obtained, which was also demonstrated by polyacrylamide gelelectrophoresis. The separation of the isozymes was interpreted as reflecting the differences in the dissociation constant for the enzyme-NADH complexes. These differences are, however, relatively small and the experiment shows the applicability of affinity chromatography for subtle separations.

Separation of the isozymes can also be achieved by ternary complex elution (unpublished observation). A complication, which affects the separation adversely, is the time-dependence of the formation

of the abortive ternary complexes.

However, ternary complex elution has been successfully utilized for the separation of the isozymes of horse liver alcohol dehydrogenase on an AMP-column (86). In this case cholic acid (an inhibitor of alcohol dehydrogenase) was added to a gradient of NAD^+ , and the SS isozyme of alcohol dehydrogenase was obtained on a preparative scale (76).

Lactate dehydrogenase isozymes from various sources have been purified by affinity chromatography on immobilized oxamate (a pyruvate analogue). The enzyme is adsorbed through ternary complex formation by addition of NAD^+ or NADH to the buffer. Pig H_4 and pig M_4 isozymes were separated and the M_4 isozyme from human placenta was purified from a crude extract by this method (87). Separation of the five isozymes of lactate dehydrogenase has not been carried out so far on an oxamate column but by a gradient elution technique this may be possible.

The isozyme X of lactate dehydrogenase, an enzyme found only in sperm, was shown not to be adsorbed to an oxamate column in the presence of NADH (88). It could thus easily be separated from the other lactate dehydrogenase isozymes which were present in an extract from mouse testes.

8. Determination of dissociation constants for complexes between enzyme and coenzyme, coenzyme fragment or coenzyme analogue (IV, 89).

The interpretation of isozyme separation led to the assumption that a correlation existed between the dissociation constant of the enzyme/eluting-ligand complex, and the eluting concentration of this ligand. In fact this has been shown for the elution of horse liver alcohol dehydrogenase from an AMP-column by different coenzymes and coenzyme fragments (*Fig. 4a*) as well as for the elution of different lactate dehydrogenases by NADH (*Fig. 4b*).

In the case of alcohol dehydrogenase the linearity was to be expected since the enzyme-immobilized ligand interaction is constant for

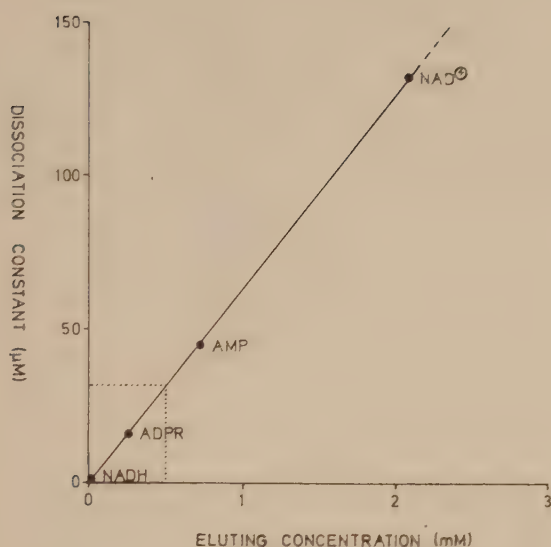


Fig. 4a. Elution of alcohol dehydrogenase from an AMP-column by different nucleotides.

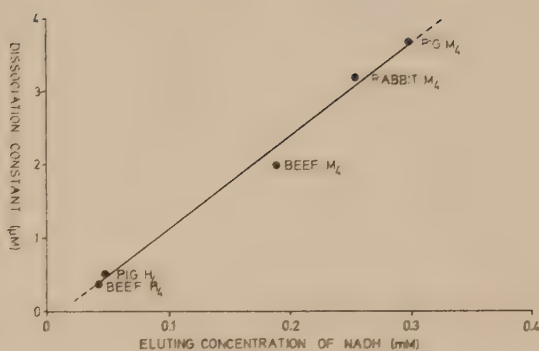


Fig. 4b. Elution of different lactate dehydrogenase isozymes from an AMP-column by NADH.

all experiments. The elution should in principle only be dependent on the interaction between eluting ligand and enzyme. After calibration of the column by eluting with ligands whose dissociation constants were known, a chromatography system was obtained which could be used for the determination of dissociation constants of complexes between alcohol dehydrogenase and coenzymes, coenzyme analogues or coenzyme fragments.

By determining the eluting concentration using N⁶-(6-aminohexyl)-5'-AMP as eluting ligand and by interpolating in Fig. 4a the dissociation constant for the enzyme-AMP-analogue complex was estimated to 32 μM. The corresponding constant for the enzyme-AMP complex is 45 μM (90). The somewhat stronger binding of the analogue can be explained by hydrophobic bonding of the spacer arm. Alcohol dehydrogenase possess a hydrophobic pocket close to the coenzyme binding site where the hydrophobic spacer arm can bind (91).

The relatively small deviations from linearity observed for different isozymes of lactate dehydrogenase (Fig. 4b) indicate that the binding of the isozymes to the immobilized AMP-analogue is quite similar.

Table 8. Estimated dissociation constants for the isozyme-NADH complexes.

isozyme	estimated dissociation constant (μM)
human H_4	0.85
chicken H_4	0.70
rat M_4	2.4

This assumption is supported by the fact that the inhibition of bovine H_4 and M_4 -isozymes by the AMP-analogue has been determined to be $0.9 \cdot 10^{-4}$ M and $0.8 \cdot 10^{-4}$ M, respectively (unpublished). Apparently, the binding of the adenosine moiety of the coenzyme to various lactate dehydrogenases is similar. The variation of the dissociation constant for the isozyme-NADH complexes could thus reflect differences in the binding of the nicotinamide moiety of the coenzyme, i.e. the nicotinamide moiety binds more tightly to the H-type of LDH. This assumption is supported by the finding that the abortive ternary LDH-NAD⁺-pyruvate complex is stronger and more tightly bound in the H_4 isozyme than in the M_4 isozyme (92).

The dissociation constants for some isozyme-NADH complexes whose values were not known were estimated by determining the concentration of NADH needed for elution and by interpolating in *Fig. 4b*. The results are summarized in *Table 8*. It should be pointed out that no difference in the dissociation constant was observed when crystalline and crude preparations of enzyme were used. Conventional methods for the determination of these constants require not only a pure enzyme but also a pure isozyme. In the present method a crude preparation, even a mixture of isozymes, can be used and obviates the necessity of a pure sample.

Other determinations of dissociation constants by affinity chromatography have been based on rather complicated theoretical considerations (93-96). This method, however, relies only on simple comparisons with conventionally determined constants. The need

for such conventionally determined constants (for calibration), however, is a limitation of the method.

AFFINITY CHROMATOGRAPHY ON IMMOBILIZED SUBSTRATE/PRODUCT.

1. Choice of ligand and immobilization of ligand.

As has been mentioned earlier the selection of a suitable ligand is difficult before some properties of the desired enzyme have been characterized. Since substrate and product are generally established a solution to the difficulty may be the immobilization of one or the other, provided that the binding strength between enzyme and ligand is strong enough to bind or at least retard the enzyme of interest. In fact, since binding can be expected to be relatively weak in many instances this could have the additional advantage that drastic elution conditions might not be necessary. With these considerations in mind a model study was carried out using α -chymotrypsin (V).

The substrate α -N-(6-aminohexyl)-L-tryptophan methyl ester was immobilized on agarose (Sepharose 4B) by two alternative methods. In the first method the spacer arm, ϵ -aminocaproic acid, was coupled to the agarose using the cyanogen bromide method and the L-tryptophan methyl ester was attached to the terminal carboxyl group of the spacer arm using a water soluble carbodiimide (resin A). In the second method the ligand was synthesized through condensation of N-carbobenzoxy- ϵ -aminocaproic acid with L-tryptophan methyl ester. After removal of the carbobenzoxy group by hydrogenolysis the ester was coupled to activated agarose in a mixture of dimethylformamide and 0.1 M NaHCO_3 (1:1), since the ester is insoluble in water (resin B). The amount of immobilized ligand was determined by different methods: for resin A by chemical hydrolysis of the agarose gel with subsequent spectrophotometric determination of liberated tryptophan or by enzymatic digestion of the substrate with excess of α -chymotrypsin under automatic titration of liberated acid, and for resin B by spectrophotometric determination of the amount of ester in the coupling solution before and after coupling. The determinations for resin A gave 3 μmol and 1 μmol of ligand per ml gel for the chemical and enzymatic determination respectively. This difference is likely to be due to the fact that not all tryptophan methyl ester is sterically available for the en-

zyme. This observation clearly illustrates the problem in determining the operative ligand concentration, which for a given column of a general ligand in fact can vary with the size of the enzyme.

2. Affinity chromatography.

Samples containing α -chymotrypsin were applied to columns of immobilized tryptophan ester. The enzyme was retarded and separated well from other proteins present in the applied sample. A slightly decreased retardation of the enzyme was observed when the column was utilized a second time. On subsequent utilization of the same column no further change in the retardation was noted. It has been shown that during the first run of the column all available substrate is hydrolysed by the enzyme. A column of immobilized product (L-tryptophan) is generated and apparently the enzyme exhibits a different affinity for this ligand.

By using a relation for the retardation on an affinity column which has been previously derived (97), a dissociation constant of approximately 6 mM was estimated for the complex between the enzyme and immobilized L-tryptophan. The corresponding constants for related compounds are: N-acetyl-L-tryptophan 17.5 mM (98) and N-nicotinyl-L-tryptophan 8.8 mM (98). Despite the relatively low affinity for the immobilized products, it is apparently sufficient to give a separation.

It has been reported that α -chymotrypsin catalyzes the hydrolysis of immobilized L-phenylalanine 4-nitroanilide (99), using a batch technique. The observation that the immobilized substrate is hydrolysed more rapidly than the soluble one is perhaps surprising. However, a 60-fold enrichment of enzyme within the gel phase was reported and interpreted to be due to affinity between enzyme and immobilized ligand. In addition to this the micro-heterogeneous distribution of substrate within the gel phase results in an apparently higher substrate concentration than in a homogeneous phase. The combination of these effects explains the fact that the hydrolysis was more rapid on the solid support. In a paper on affinity chroma-

tography of heavy meromyosin subfragment-1 (a protein with ATPase activity) on an ATP-column the following conclusion was drawn: "under condition in which bound ATP is split, the protein is adsorbed not only to intact ATP, but also to the decomposition products of the latter" (i.e. immobilized ADP) (100).

All these observations support the assumption that the retardation of α -chymotrypsin is due to interactions with the generated product. In summary, a simple preliminary approach to affinity chromatographic purification, especially of new enzymes, can in many cases involve the immobilization of either enzyme substrate or product. Since turnover of substrate occurs, direct coupling of the product may be the most rational choice, thus utilizing the effect of product inhibition in affinity chromatography.

CONCLUDING REMARKS.

The results presented in this thesis as well as in other investigations clearly illustrate the applicability of general ligands in affinity chromatography. For the purification and separation of enzymes the development of specific elution methods has been most valuable.

The potential of general ligands as a tool for studies on enzyme mechanisms and kinetics has so far only been indicated. Further investigations of this kind will give information on enzyme-ligand interactions. In this context the use of differently substituted adenosine analogues may give information about the enzyme-coenzyme interaction. Furthermore, the coenzyme specificity may be established by comparative studies on different analogues.

The utilization of immobilized substrate/product has been shown to be a possible preliminary approach to the purification of enzymes by affinity chromatography. However, the enzyme-ligand interaction is in many cases weak, with the result that the enzyme is only retarded on the column. A drawback in such cases is the impossibility of concentrating a dilute enzyme solution, which is one of the inherent advantages of affinity chromatography.

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